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UTILIZATION OF FIBROUS WASTES AS SOURCES OF NUTRIENTS

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The purpose of this study was to develop a biological system for conversion of natural cellulosic wastes to products that can be utilized as nutrients by animals. Efforts toward development of a fermenter for the growth of an anaerobic cellulolytic bacteria on cellulosic materials were not successful because the yield of bacterial cells and other products was too low. However, a repression mechanism was demonstrated on the synthesis of cellulose that prevents accumulation of sugar from the hydrolysis of cellulose. The strictly anaerobic bacteria Ruminococcus albus was successfully grown on petri plates. Trichoderma viride cultures also were used in laboratory experiments for degradation of cellulose.

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FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment--air, water, and land. The National Environmental Research Centers provide this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on man and the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

In an attempt to solve the problems involved in solid waste disposal, this study is concerned with developing a biological system for the conversion of natural cellulosic wastes to products that can be utilized as nutrients by animals.

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Introduction

The specific aim of the research for the total grant period was to develop a biological system for the conversion of natural cellulosic wastes to products that can be utilized as nutrients by animals.

Initial efforts were toward the development of fermenter for the growth of an anaerobic cellulolytic bacteria on cellulosic materials. Bacterial cells and products of fermentation, including sugars, were the expected nutrients. The yield of cells and other products were too low. Different types of fermenters with various changes in operational procedures were not successful.

We demonstrated a repression mechanism on the synthesis of cellulase which prevents accumulation of sugars from the hydrolysis of cellulose (Fusee and Leatherwood, 1972). A major effort subsequently was directed at changing the microorganism by mutation in order that a more rapid rate and extent of cellulose hydrolysis could be achieved. An anaerobic glove box was designed and built primarily for the mutation studies. We were successful in growing the strictly anaerobic bacterium, Ruminococcus albus on petri plates. However, due to the difficulty of operation, the mutation studies of the anaerobic bacteria were postponed. We recently initiated mutation studies with aerobic and facultative cellulolytic bacteria.

A. Use of Trichoderma viride enzyme in the degradation of newsprint.

Trichoderma viride is one of the most potent cellulolytic fungi. Long-term incubations of cotton linters with culture filtrates of T. viride resulted in the degradation of about one-half of the cellulose. The following experiment was designed to yield nutrients from newsprint digested by the enzymes in T. viride culture in a natural silo-type of anaerobic fermentation.

Procedure

T. viride QM6a was obtained from ATCC and grown on a synthetic-cellulose medium similar to that described by Mandels. Cultures of rapidly growing T. viride were used to inoculate 20 l. carboys containing 10 l. of medium. These cultures were incubated at room temperature with aeration for 18 days. Subsequent to adding the cultures to the newsprint in the fermenter, the enzymatic activity and contamination by other microorganisms were determined.

The fermenter consisted of a large plastic tank (250 l capacity) with a lid which was fitted with a gas-relieve valve and a valve in the bottom of the fermenter for collecting liquid. Newsprint and T. viride cultures were added to the fermenter to produce a wet mass on the dates shown in Table 1. The newsprint (News and Observer, Raleigh) was unprinted and had been shredded in a hammer mill equipped with a large mesh screen.

Cellulolytic activity was determined in 0.1 ml of the supernatant liquid of both the cultures prior to addition to the fermenter and the liquid samples and product collected from the fermenter. The assay consisted of reacting the enzyme with 2.0 ml of 1% carboxymethylcellulose (CMC4L, Hercules Powder Co., Dela.) at pH 4.5 and 55°C for 60 minutes. Reducing groups were measured in the enzyme assay at zero time and 1 hr. by the dinitrosalasylic acid reagent (DNS, Sumner 1944). Relative activity is reported as the absorbance change of the DNS due to 0.1 ml of enzyme.

Samples of collected liquid product were analyzed for pH and dry matter. Volatile fatty acids were determined by gas-liquid chromatograph (GLC) on an SP 1200 column containing 1% H_3PO_4 (Supelco.). The samples were prepared for GLC by treating 5 ml of fermenter product with 1 ml of 25% metaphosphoric acid.

Results and Discussion

Cultures of T. viride varied in the amount of cellulolytic activity in the supernatant liquid. Contamination of the cultures with bacteria reduced the level of enzyme activity. Eight carboys of media were inoculated simultaneously. The results of the relative enzyme level of each carboy of culture is shown in Table 2.

Facilities were not available for growing cultures in sufficient volume to fill the fermenter. Therefore, sequential addition of cultures and newsprint approximately on a three-week interval was accepted as the best alternative plan. Attempts were made to keep the newsprint-cultures in a stratified layer in order to collect material from the bottom of the fermenter which had been incubated the maximum time.

Collections of representative samples of the fermenter liquid were made after withdrawing through the bottom valve 1 liter of liquid which was added to the top layer in the fermenter. Subsequently 200 ml samples were collected for analyses. The results of analyses of both samples and large batch collections of fermenter effluents is shown in Table 3 and 4.

Approximately 25 to 30% of the cellulolytic activity added in the cultures to the fermenter was collected in the fermenter effluents. A trend of an increase in enzyme recovery was apparent with increased incubation time. This effect would be expected since it is well known that cellulolytic enzyme has a high adsorptive affinity for its substrate. Thus as the cellulose becomes saturated with respect to adsorptive sites more enzyme would remain unadsorbed.

The pH of samples removed from the fermenter were indicative of the pH of natural forage fermentations in silos. However, removal of large batches of fermenter effluents revealed pH values much lower than anticipated. Apparently, lactic acid, one of the more acidic organic acids which accumulate in mixed anaerobic fermentations is present in considerable quantities

in the fermenter effluents. The presence of large quantities of organic acids in the fermenter effluents also is indicated by the large amounts of NaOH (5g/1.) necessary to neutralize the fermenter effluents to pH 7.5. Analyses of fermenter effluents for lactic acid, succinic acid and long chain fatty acids currently is being conducted. Dry matter values as reported in Table 3 are not indicative of the dry matter yield of the fermenter since these determinations were made by lypholizing the acid effluents. Under these conditions the volatile acids, aldehydes and alcohols should be vaporized from the sample. The heat of combustion was determined in a Parr bomb calorimeter. Samples lypholized for dry matter determinations were subsequently combusted. The losses of volatile compounds would be expected in the drying process, thus the heat of combusion is representative of only the non-volatile energy compounds. The values of heat of combusion indicate that only about one-half of the dry matter is combustible organic matter.

In addition to the above analyses, anthrone sugars, reducing sugars and protein measured on the basis of absorption of light in the 260 and 280 nm spectrum was determined. These latter values apparently are not significantly great. This indicates that soluble carbohydrates and proteins are not accumulating in the fermenter liquid as had been anticipated.

If a fermenter system is to be effective in the degradation of cellulose to yield nutrients then a system must be devised for a minimum of operational expense. We found that a large volume of precipitate formed when the liquid from the fermenter was adjusted to pH 7.5 with NaOH. Collection of this precipitate can be accomplished by allowing it to settle and by means of centrifugation of the concentrated precipitate. The precipitate yielded after lypholizing 34 and 26 grams of dry powder from the two large batches, respectively, which has an apparent protein content of 27%.

Experiments are now in progress to determine the feasibility of using the neutralized supernatant liquid as a major source of nutrients for the growth of the fungi. This process would permit recycling of mineral nutrients through the cultures and fermenter.

TABLE 1.

Schedule followed in adding newspring and T. viride culture to the fermenter and collecting of liquid products.

Date	Material	Amount
May 15, 1972	added	
	Newsprint	4.0 kg
	T. viride culture	60 l.
June 6, 1972	added	
	Newspring	4.0 kg
	T. viride culture	60 l.
June 26, 1972	added	
	Newsprint	5.5 kg
	T. viride culture	
July 17, 1972	collected - sample	200 ml
July 24, 1972	collected - sample	200 ml
August 4, 1972	collected - sample	200 ml
August 14, 1972	added	
	T. viride culture	60 l.
	collected - product	60 l.
September 5, 1972	added	
	Newsprint	4.0 kg
	T. viride culture	70 l.
	collected product	70 l.

TABLE 2.

Relative enzyme activity of T. viride cultures grown in carboys.

Culture No.	Relative Activity				
	May 15	June 6	June 26	August 14	September 5
1	0.20*	0.10*	0.31*	0.74	0.49
2	0.77	0.08*	0.04*	0.76	1.12
3	0.72	0.52	0.38	0.73	0.66
4	0.03*	0.58	0.41	0.96	0.38
5	0.38	0.42	0.40	0.22*	0.01*
6	0.81	0.36	0.39	1.22	0.82
7	0.46	0.37	0.43	0.70	0.70
8	0.76	0.33	0.36	1.23	0.60
Avg [A]	0.65	0.43	0.40	0.91	0.68

* Represents those cultures which were not added to fermenter because of low activity or contamination.

[A] Average relative activity of cultures added to fermenter

TABLE 3.

Enzymatic activity, pH, dry matter and heat of combustion of liquid collected from the fermenter.

Date	Volume liters	Relative Enzyme Activity	pH	Dry Matter %	KCal/g
July 17	0.2	.12	4.18	0.7	2.2
July 24	0.2	.14	4.18	0.8	2.0
August 4	0.2	.17	4.17	0.7	1.7
August 14	60.	.20	3.80	0.8	1.9
September 5	70.	.16	3.87	0.7	1.4

TABLE 4.

Volatile fatty acid content of liquid material collected from the fermenter.

Date	Acetic Acid %	Butyric Acid %	Unknown [A] %
July 17	0.66	0.40	0.40
July 24	0.60	0.40	0.43
August 4	0.88	0.56	0.43
August 14	0.77	0.67	0.25
September 5	1.22	0.67	0.16

[A] The unknown volatile fatty acid had a retention time equal to that of C-8 monocarboxylic acid.

B. Effects of treatment of cotton linters on cellulose hydrolysis by Ruminococcus albus.

A major problem encountered in the effective utilization of microbial fermentation of cellulosic materials is the slow rate and limited extent of hydrolysis. In 1969, a new mechanism of cellulose hydrolysis was presented (Leatherwood, 1969). This new mechanism allowed that resistant cellulose residue and slow rate of hydrolysis were associated with the number of available binding sites on the insoluble cellulose.

Callihan and Dunlap (SW-24c) and many other researchers have reported that alkali and/or other treatments of cellulosic materials significantly increased the rate and extent of cellulose digestion. This experiment was designed to measure the effect of treatment of cotton linters, a very resistant cellulose, with either delute acid, dilute alkali or trypsin on the extent of cellulose hydrolysis. The remaining residue after exposure to growing cells of Ruminococcus albus was retreated.

Procedure

Cotton linters (15 g) from Buckeye Cellulose Corporation was treated with either 0.1 N HCl, 0.1 N NaOH, 0.05 mg/ml of trypsin in phosphate buffer pH 7.0 or water (control) for 1 hr at room temperature. The slurry (500 ml) was poured into a glass column (8 x 36 cm) fitted with a sintered glass filter. The cellulose residue was repeatedly washed in the column with distilled water until the effluent was approximately neutral. The control and trypsin treated samples were washed with similar volumes of water. All samples subsequently were washed three times with acetone and once with ether. The residue was dried and added to 3 liter round bottom flasks which were used to prepare 1500 ml of liquid medium for Ruminococcus albus as described in previous report. The flasks were innoculated and incubated for 20 days. The cellulose residue remaining was collected and retreated by the procedure

described. The treated residue was again used as a substrate in growing cultures of R. albus. This process was repeated twice.

Results

Growth of the cellulolytic bacteria on the treated cotton linter residue was slow. The extent of cellulose digestion was low and variable. The results are shown in Table 1.

Table 1. Digestion of cotton linters by Ruminococcus albus after treatment with either dilute acid, dilute alkali, trypsin, or water (control).

	% Digestion			
Growth period	1	2	3	Total
Treatment				
0.1 N HCL	17.7	3.2	-	20.9
0.1 N NaOH	10.3	0.5	1.4	12.2
0.05 mg/ml Trypsin	9.4	1.4	1.0	11.8
water (control)	6.1	3.7	.6	10.4

Although dilute acid treated cellulose residue was digested to the greatest extent, the effect of treatment does not appear to be significant. Bryant and Halliwell have reported the digestion of untreated cotton fibers to be only about 10% by Ruminococcus albus strain 7. It is strange that the same cotton linters as used in this experiment apparently are hydrolized completely in dilute concentrations in semisolid agar medium.

Conclusions and Recommendations

Incorporation of other bacteria along with the cellulolytic bacteria generally improves the rate and extent of cellulose digestion as shown in our work and by Callihan and Dunlap. This phenomenon maybe due to the removal of intermediary products that cause catabolite repression of cellulase synthesis similar to the effect shown by Fusee and Leatherwood, 1972. One way of improving fermenter yield may be the use of specific combinations of mixed bacterial cultures.

Glucose and cellobiose do not accumulate in growing cultures of cellulolytic microorganisms. Apparently a repression mechanism prevents synthesis of excess amounts of cellulase. Cellobiose does not inhibit either the hydrolytic activity of R. albus on carboxymethylcellulose or the adsorption of hydrolytic activity to cellulose. However, the addition of cellobiose to growing cultures of R. albus does inhibit the solubilization of cellulose. The control of cellulase synthesis probably occurs through a regulator-operon system. We attempted to produce a mutant that would produce constitutive cellulase and thus would accumulate sugars. Our extensive efforts to produce a mutant with these characteristics were not successful. However, to maximize fermenter yields, mutation and selection of the proper organisms will be necessary.

Extent of cellulose hydrolysis is greater in semi-solid agar medium than in liquid medium. The reasons for this variation due of the physical characteristics of the media needs to be elucidated so that fermenter yields can be maximized.

References

Fusee, M. C. and J. M. Leatherwood 1972. Regulation of cellulase from Ruminococcus. Canadian J. Microbiol. 18:347-353.

Halliwell, G. and M. P. Bryant. 1963. The cellulolytic activity of pure strains of bacteria from the rumen of cattle. J. Gen. Microbiol. 32:441-448.

Leatherwood, J. M. 1969. Cellulase complex of Ruminococcus and a new mechanism for cellulose degradation, p. 53-59. In Robert F. Gould (ed.), Cellulases and their applications, Advances in chemistry series 95. American Chemical Society, Washington.